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ORIGINAL ARTICLE

Survival of *Escherichia coli* in cowpats in pasture and in laboratory conditions

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Abstract

Aims: To compare survival of *Escherichia coli* and faecal coliforms (FC) in bovine faeces deposited in a pasture or incubated in a controlled laboratory environment at temperatures within the same range.

Methods and Results: Faecal samples from three cow herds were deposited as shaded and nonshaded cowpats in a field and incubated in a laboratory for one month at 21·1, 26·7 and 32·2°C. Both FC and *E. coli* concentrations increased as much as 1·5 orders of magnitude both in the field and in the laboratory during the 1st week and subsequently decreased. In shaded cowpats, the die-off of *E. coli* and FC was significantly slower, and the proportion of *E. coli* in FC was significantly larger as compared with nonshaded cowpats. The die-off was faster in the field than in the laboratory at similar temperatures.

Conclusions: FC and *E. coli* die-off rates were substantially lower in laboratory conditions than in the field within the same range of temperatures.

Significance and Impact of the Study: This study underscores the importance of field data on survival of manure-borne FC and *E. coli*, and indicates that laboratory die-off rates have to be corrected to be used for field condition simulations.

Introduction

In recent years, there has been substantial interest in the transport and fate of faecal-borne bacteria in the environment. The US EPA has been tasked with identifying impaired surface waters because of excessive faecal contamination, and developing strategies for mitigating contamination. Animal waste is recognized as an important source of faecal contamination in the environment. Consequently, a thorough understanding of environmental factors responsible for the survival of faecal-borne bacteria is critical for evaluating management practices aimed at decreasing bacterial contamination of water from land-applied manures or faecal deposition.

Faecal coliform (FC) bacteria have been historically used as indicators of faecal contamination. However, there is growing recognition that many common water-borne FC; e.g., *Klebsiella*, *Enterobacter*, *Citrobacter*, are not necessarily of faecal origin (Doyle and Erickson 2006). Consequently, the EPA now recommends the use of *Escherichia coli* as a more reliable method for identifying impaired streams and water bodies (U.S. Environmental Protection Agency 2001). This is also consistent with the fact that *E. coli*, including pathogenic strains (e.g., enterohaemorrhagic *E. coli* O157:H7), are the predominant FC present in mammalian faeces. However, considering the extensive databases which exist for water-borne FC, an understanding of both FC and *E. coli* survival will be useful in evaluating best management practices.

Numerous factors have been documented affecting the survival of manure-borne *E. coli*, including temperature, moisture content, pH, diet of animals, strain, UV or solar radiation and type of soil (Crane and Moore 1986; Crane *et al.* 1984; Rosen 2000; Guan and Holley 2003; Larney *et al.* 2003; Unc and Goss 2003; Wang *et al.* 2004). However, relatively little is known about the die-off rates of FC or *E. coli* in animal faeces deposited on pasturelands, or the relative importance of the different environmental factors. Avery *et al.* (2004) found that the average rate of decline for *E. coli* in deposited cattle faeces was substantially lower than in pig faeces. Based on field studies, Buckhouse and Gifford (1976) and Bohn and Buckhouse (1985) suggested that cattle faeces could provide a protective medium in which coliforms could survive for at least a year. Kress and Gifford (1984) and Thelin and Gifford (1983) described a nonlinear effect of cowpat age on the amount of FC available for rainfall-induced release to the soil. Meays *et al.* (2005) showed that age of faecal pats, as well as exposure to solar radiation, negatively influenced the survival of *E. coli*, whereas the moisture content of faecal pats at sampling time was not correlated with the numbers of *E. coli* present. Laboratory studies have demonstrated the effects of temperature and initial bacterial inoculum on survival of bovine faeces-borne *E. coli* O157:H7 (Wang *et al.* 1996; Kudva *et al.* 1998; Bach *et al.* 2005).

Comparisons of field and laboratory survival data have produced contradictory results. Avery *et al.* (2004) noted that the *E. coli* die-off in field studies was slower than in the laboratory study of Himathongkham *et al.* (1999). They indicated that this might be because of *E. coli* strain/serotype differences, or to unknown factors that influence the fate of *E. coli* in freshly voided faeces. Bolton *et al.* (1999) compared survival of nontoxigenic strains of *E. coli* O157 in bovine faeces in laboratory and field conditions and observed a more rapid die-off in exposed samples.

The overall objective of this work was to investigate the survival of *E. coli* and FC in bovine faecal cowpats deposited on pasture *vs* those incubated in a controlled laboratory environment at comparable average temperatures.

Materials and methods

Faecal sample collection and placement

Faecal samples were obtained from three herds – 20-month-old beef heifers and mature beef cows (Angus and Hereford), and dry dairy (Holsteins) cows – grazing on pasture with the predominant species being tall fescue (*Lolium arundinaceum*), Kentucky blue grass (*Poa pratensis*) and white clover (*Trifolium repens*). To obtain sufficient faeces for all treatments, faecal samples were obtained via rectal palpation from 10 to 15 cows in each of the three herds before 9:00 a.m. on 20 June 2004. Samples were combined within herds.

Pasture. Six composite faecal samples (600 g) were made for each herd. A total of nine samples, i.e., three samples per herd, were placed at a site open to the sun on a 3×3 grid with 1-m distance between cowpats using a randomized design. Another nine samples were similarly placed in shaded area under a tree. Faecal samples for future sampling were poured into rings (15 cm ID $\times$ 2 cm height) put on the ground. An additional cowpat was put in both the sun and shade sites for temperature measurements. A temperature probe was placed directly in the cowpat with a temperature-logging device (HOBO Temp, Onset Computer Corporation, Cape Cod, MA, USA), and temperature was recorded on a bi-hourly basis. A weather station located approx. 0·5 km away was used to obtain hourly air temperature and rainfall data. Two rain gauges were installed on the site and checked daily.

Laboratory. The 250 ml samples were placed in pans in controlled environment chambers (Conviron, CMP4030, Controlled Environment Limited, Winnipeg, CA, USA) and covered with punctured plastic film. The samples were incubated at 21·1, 26·7 and 32·2°C. There were three samples for each herd at each temperature.

Sampling schedule and analysis

Cowpats in the field and laboratory were subsampled on days 0, 4, 8, 12, 16, 20 and 27. A 20-g sample was taken using a spatula. Subsamples were divided into two equal parts; one part was used to measure gravimetric water content by drying at 105°C for 72 h and the second part was used for microbiological analysis. All analyses were carried out on the sampling day.

Faecal coliforms. Samples were blended in a table-top blender for 2 min, transferred to a 15-ml conical test tube and centrifuged for 15 min at 100 ***g***. Supernatants were diluted (1 : 100) by sterile transfer of 110 μl to a tube containing 1-ml sterile water. The diluted samples were spirally plated (50 μl; Autoplate 4000; Spiral Biotech, Norwood, MA, USA) in duplicate onto MacConkey Agar plates and incubated at 44°C for 24 h. Red (lactose fermenting) colonies were counted with an automatic colony counter (Q Count, Spiral Biotech, Inc., Norwood, MA, USA).

Escherichia coli. After enumeration of FC, isolated, red colonies (25 to 50 per plate) were transferred from MacConkey Agar plates in a grid pattern onto MacConkey Agar, Simmons Citrate Agar and L-Agar (Lennox Broth base with 1·5% agar; Gibco Laboratories, Long Island, NY, USA) plates and incubated at 37°C for 24 h. Colonies that fermented lactose (pink) on MacConkey agar, but did not ferment citrate, and exhibited no swarming on L-agar were scored as *E. coli*. *Escherichia coli* concentrations were determined by multiplying the total FC count by the proportion of FC as *E. coli*.

Statistical analysis

The statistical software package SPLUS 2000 (Mathsoft, Cambridge, MA, USA) was used to perform analysis of variance and paired comparisons between adjusted means in treatments. Statistics were computed for natural log-transformed *E. coli* and FC contents, for logit-transformed values of proportions of *E. coli* in FC and for nontransformed moisture contents. The die-off rate constants were obtained from the slopes of linear regressions of time *vs* logarithms of bacteria concentrations; changing sign of the slope gave the die-off rate constant.

Results

Pasture

Weather and cowpat temperature data are shown in Fig. 1a. The average air temperature during the observation period was 25·8°C. At 8 a.m., open cowpats were on average 3°C warmer than the shaded cowpats. A preliminary month-long study with cowpats from the beef herd showed that the temperature of shaded cowpats during the day was lower than the air temperature when cowpat water contents were above 50%. The regression line was $T_{cowpat} = 0{\cdot}452\ T_{air} + 12{\cdot}2$ with the determination coefficient $R^2 = 0{\cdot}834$ where T is the temperature,°C. Shaded cowpats received 70% to 80% less rain than those exposed to the sun. The initial water contents of cowpats on pasture were about 90% for all three herds (Table 1, Fig. 1b). Water contents generally decreased over time and were between 50% and 70% at the end of observation period. Rain events caused an increase in water contents between days 12 and 16 and days 20 and 27. Water contents in shaded cowpats were about 3% lower than in open cowpats ($P = 0{\cdot}065$).

Initial (day 0) *E. coli* concentrations in the fresh cowpats varied from 10^6 to 10^7 g^{-1} dry weight (Table 1). *Escherichia coli* concentrations in cowpats over time are shown in Fig. 1c. There was a substantial (up to 1·5 log) growth of *E. coli* during the first 6 to 8 days, followed by a steady decline in viability; although temporary increases in concentrations were observed between days 12 and 16 and days 20 and 27 corresponding to rainfall events (Fig. 1c). The concentrations dropped below initial concentrations in all cowpats only on day 20. Overall, *E. coli* concentrations decreased less than two orders of magnitude during the month of observations. *Escherichia coli* concentrations in shaded cowpats were on average about 2·2 times higher than open cowpats ($P = 0{\cdot}008$).

At the beginning of the incubations and during the initial growth phase, the FC population consisted exclusively of *E. coli* (Fig. 1d). Concomitant with a decrease in FC populations (after day 8), the proportion of *E. coli* in FC also decreased; although temporary increases in *E. coli* proportions were observed between days 12 and 16 and days 20 and 27 corresponding to rainfall events (Fig. 1d). Proportion of *E. coli* in FC was on average about 0·16 higher in shaded than in open pats ($P = 0{\cdot}002$). The decrease in FC concentrations was slower than that of

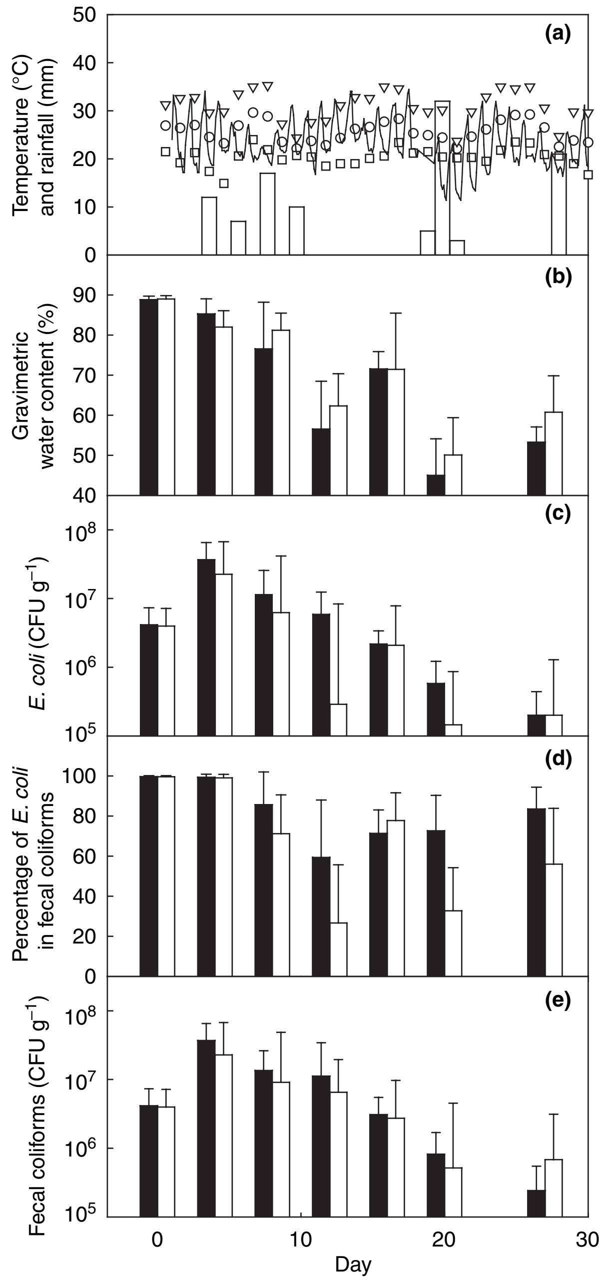

Figure 1 Field experimental data. Panel a – temperature and rainfall; ▽, maximum; ○, average and □, maximum daily temperature; bars, daily rainfall; line, shaded cowpat temperature; panel b – cowpat water content, filled and hollow bars in this and subsequent panels show data for tree-shaded and open to direct sun cowpats, respectively, panel c – *Escherichia coli* concentrations, panel d – percentage of *E. coli* in faecal coliforms, panel e – faecal coliform concentrations; error bars represent standard errors.

Table 1 Faecal coliform content, moisture content and pH of bovine faeces

Herd	pH	Log (Faecal coliforms, CFU g^{-1})	Water content, mass%
Beef cows, 20 month	7·17 ± 0·04	6·24 ± 0·13	90·4 ± 0·1
Beef cows 32 month	6·87 ± 0·21	6·78 ± 0·14	89·0 ± 0·2
Dry dairy cows	7·66 ± 0·07	6·73 ± 0·04	87·8 ± 0·1

Average ± standard error from triplicate measurements.

E. coli (Fig. 1e), and unlike *E. coli*, FC concentrations were not affected by shading ($P = 0·52$).

Laboratory conditions

Moisture content of cowpats did not change significantly during the observation period. Average water contents in faeces from the three studied herds were 89%, 90% and 87%, respectively.

Escherichia coli concentrations in faeces incubated in the laboratory over time are shown in Fig. 2a. *Escherichia coli* population dynamics in faeces incubated under laboratory conditions generally demonstrated a 'growth followed by die-off' pattern similar to the one observed under field conditions. Similar to field conditions, during the initial growth phase the FC population consisted exclusively of *E. coli*, followed by a decrease in the proportion of *E. coli* during the die-off phase (Fig. 2b). As the temperature increased, a larger percentage of the FC were species other than *E. coli* ($P < 0·001$).

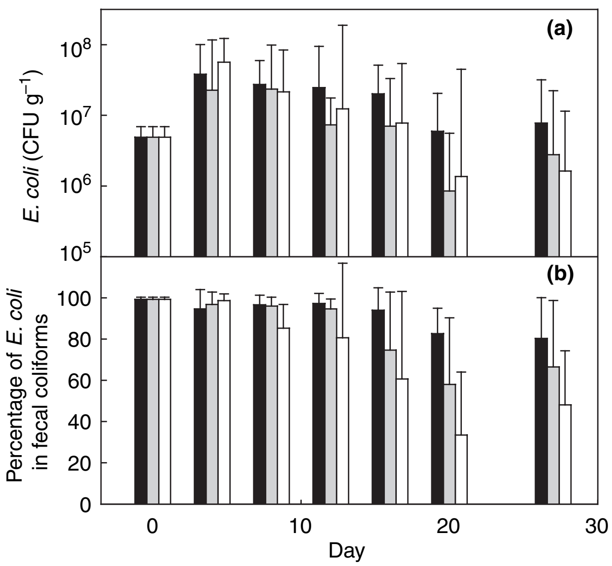

Figure 2 Laboratory experimental data. Panel a – *Escherichia coli* concentrations, panel b – percentage of *E. coli* in faecal coliforms. ■, 21·1°C, ▩ 26·7°C, □ 32·2°C.

Die-off rate constants

The die-off rate constants for *E. coli* and FC (Table 2) were computed using data starting from day 4. The field *E. coli* die-off rate constants were not significantly different between the shaded and exposed cowpats. The field die-off rates of *E. coli* and FC were not significantly different. Rate constants in laboratory incubated samples were substantially smaller than the rate constants in field incubated samples (Table 2). Laboratory *E. coli* die-off rates tended to be larger than those of FC, although the differences were not statistically significant.

Discussion

Based on the results of both the field and the laboratory portions of this study, it appears that cowpats may remain a substantial source of *E. coli* for at least 30 days after deposition and maybe much longer, mostly because of a substantial growth in *E. coli* during the first 4–8 days (Figs 1 and 2). Clemm [(1977), cit. from Kress and Gifford 1984] and Muirhead *et al.* (2005) found that there was an initial increase in the number of bacteria in defecated cow faeces for the first 2 weeks, and by the 5th week the bacteria were back to their initial levels. Wang *et al.* (2004) observed an initial growth of approx. two orders of magnitude of *E. coli* and FC populations at 27°C in bovine faeces. Initial growth was also observed in one of two trials at 4°C in the work of Wang *et al.* (2004). Avery *et al.* (2004) did not observe an initial *E. coli* population growth over the first 14 days in their field study with temperatures between 0·4 and 15·6°C recorded in soil under the deposited faeces. An initial one-order of magnitude growth was observed in data of Kudva *et al.* (1998) on survival of *E. coli* O157:H7 in bovine faeces at 22 and 37°C but not at −20, 4, 45 and 70°C. Similarly, Bach *et al.* (2005) observed a one-order of magnitude growth

Table 2 Average ± standard error die-off rate constants (per day) of *E. coli* and faecal coliforms in cowpats after the day 4

	Field		Laboratory		
Micro-organisms	Open	Shade	21·1°C	26·7°C	32·2°C
E. coli	0·205 ± 0·070	0·230 ± 0·012	0·080 ± 0·020	0·125 ± 0·044	0·166 ± 0·028
Faecal coliforms	0·225 ± 0·017	0·169 ± 0·030	0·071 ± 0·018	0·103 ± 0·039	0·125 ± 0·016

of the *E. coli* O157:H7 population in bovine faeces during the first 2 weeks of incubation at 22°C, but not at 4 or −10°C. We concur with the conclusion of Wang *et al.* (2004) that 'as-excreted' estimates of manure bacterial populations may underestimate populations available for contamination of surface runoff for several days post-excretion. The above references indicate that temperature seems to be the leading factor affecting the magnitude of the initial growth of the *E. coli* population in freshly deposited bovine faeces. The range of temperatures between 20 and 35°C appeared to be the most favourable for the post-deposit growth.

Shading somewhat decreased *E. coli* die-off rates in cowpats (Fig. 1c and its discussion above). Meays *et al.* (2005) studied *E. coli* die-off in cowpats shaded to different extents and concluded that moisture content was not a very useful covariate. However, they considered the period of 45 days that included an initial growth up to day 7 in nonshaded samples and substantial die-off of *E. coli* in nonshaded samples on day 31 and later. Moisture content decreased between days 7 and 31 in concert with the decrease in *E. coli* population in 100% shaded and exposed samples in the work of Meays *et al.* (2005). This is the period of time in our study where shading had a significant affect on cowpat moisture content. More work is needed to define the environmental covariates that are useful in estimating *E. coli* die-off rates.

Ratios of estimated *E. coli* die-off constants at 32·2 and 21·1°C were about two (Table 2). The Arrhenius equation $k_1/k_2{=}\theta^{T_1-T_2}$ has been suggested as a way to relate the rate constants k_1 and k_2 at temperatures T_1 and T_2 respectively. The value of θ has been typically set at about 1·07 in various environments including manure (Pachepsky *et al.* 2006). With such a value of θ, the prediction of the die-off constants at 21·1 and 32·2°C from the Arrhenius equation ratio is 2·12, which is close to the average of the ratios (2·07) found in our experiments (Table 2). For FC, the ratios were lower and corresponded to θ = 1·05.

In all experiments, *E. coli* accounted for 100% of the FC populations in the original faeces and during the growth phase. During the die-off phase, the proportion of *E. coli* decreased to between 50% and 85% (P = 0·95) after 27 days under both pasture and laboratory conditions. Our data generally agree with those of Wang *et al.* (2004), who observed *E. coli* proportions of 75% and 45% on days 32 and 35, respectively, for two different experiments. Their experiments encompassed water contents ranging from 30% to 80%; the proportion of *E. coli* decreased faster at 30% than at higher moisture contents. These results indicate *E. coli* are dying off more rapidly than non-*E. coli* FC. Consequently, *E. coli* are the predominant faecal-borne bacteria responsible for microbial contamination of water at early stages of manure dissolution, although non-*E. coli* FC maybe responsible for limited contamination as faeces/manure age.

Overall, comparison of laboratory and field data shows that the factors affecting die-off manifested themselves similarly in both environments, although die-off rates were different. There is a need for more field data documenting *E. coli* die-off rates, and elucidation of factors controlling the rates. Collection of additional laboratory data, where individual factors can be more easily defined and controlled, will also be critical for improving models to describe and predict these interactions.

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References

Avery, S.M., Moore, A. and Hutchison, M.L. (2004) Fate of *Escherichia coli* originating from livestock faeces deposited directly onto pasture. *Lett Appl Microbiol* **38**, 355–359.

Bach, S.J, Stanford, K. and McAllister, T.A. (2005) Survival of *Escherichia coli* O157:H7 in feces from corn- and barley-fed steers. *FEMS Microbiol Lett* **252**, 25–33.

Bohn, C.C. and Buckhouse, J.C. (1985) Coliforms as an indicator of water quality in wildland streams. *J Soil Water Conserv* **40**, 95–97.

Bolton, D.J., Byrne, C.M., Sheridan, J.J., McDowell, D.A. and Blair, I.S. (1999) The survival characteristics of a non-toxigenic strain of *Escherichia coli* O157:H7. *J Appl Microbiol* **86**, 407–411.

Buckhouse, J.C. and Gifford, G.F. (1976) Water quality implications of cattle grazing on a semiarid watershed in southeastern Utah. *J Range Management* **29**, 109–113.

Clemm, D.L. (1977) Survival of bovine enteric bacteria in animal wastes. *Masters Thesis*. Central Washington University, Ellensburg, WA, USA.

Crane, S.R. and Moore, J.A. (1986) Modeling enteric bacterial die-off: a review. *Water Air Soil Pollut* **27**, 411–439.

Crane, S.R., Moore, J.A., Grismer, M.E. and Miner, J.R. (1984) Bacterial pollution from agricultural sources: a review. *Trans Am Soc Agric Eng* **26**, 858–866.

Doyle, M.P. and Erickson, M.C. (2006) Closing the door on the fecal coliform assay. *Microbe* **1**, 162–163.

Guan, T.Y. and Holley, R.A. (2003) Pathogen survival in swine manure environments and transmission of human enteric illness – a review. *J Environ Qual* **32**, 383–392.

Himathongkham, S., Nuanualsuvan, S., Riemann, H. and Cliver, D.O. (1999) Survival of *Escherichia coli* O157: H7 and *Salmonella typhimurium* in cow manure and cow manure slurry. *FEMS Microbiol Lett* **178**, 251–257.

Kress, M. and Gifford, G.F. (1984) Fecal coliform release from cattle fecal deposits. *Water Resource Bull* **20**, 61–66.

Kudva, I.T., Blanch, K. and Hovde, C. J. (1998) Analysis of *Escherichia coli* O157:H7 survival in ovine or bovine manure and manure slurry. *Appl Environ Microbiol* **64**, 3166–3174.

Larney, F.J., Yanke, L.J., Miller, J.J. and McAllister, T.A. (2003) Fate of coliform bacteria in composted beef cattle feedlot manure. *J Environ Qual* **32**, 1508–1515.

Meays, C.L., Broersma, K., Nordin, R. and Mazumderd, A. (2005) Survival of *Escherichia coli* in beef cattle fecal pats under different levels of solar exposure. *Rangeland Ecol and Mngmt* **58**, 279–283.

Muirhead, R.W., Collins, R.P. and Bremer, P.J. (2005) Erosion and subsequent transport state of *Escherichia coli* from cowpats. *Appl Environ Microbiol* **71**, 2875–2879.

Pachepsky, Y.A., Sadeghi, A.M., Bradford, S.A., Shelton, D.R., Guber, A.K. and Dao, T. (2006) Transport and fate of manure-borne pathogens: modeling perspective. *Agric Water Mngmt* **86**, 81–92.

Rosen, B.H. (2000) *Waterborne Pathogens in Agricultural Watersheds*. USDA NRCS: Watershed Science Institute, 62 p.

Thelin, R. and Gifford, G.J. (1983) Fecal coliform release patterns from fecal material of cattle. *J Environ Qual* **12**, 57–63.

U.S. Environmental Protection Agency. (2001) *Protocol for Developing Pathogen TMDLs*. EPA 841-R-00-002Office of Water (4503 F) Washington, DC: United States Environmental Protection Agency.

Unc, A. and Goss, M.J. (2003) Impact of manure properties on the survival of manure *Escherichia coli* in soils. In *Proceedings of 2nd Canadian Organic Residuals Recycling Conference* 24–25 April 2003. ed. Kelly, H. and Bryden, J., pp. 331–346, Penticton, BC, Canada.

Wang, G., Zhao, T. and Doyle, M. (1996) Fate of enterohemorrhagic *Escherichia coli* O157:H7 in bovine feces. *Appl Environ Microbiol* **62**, 2567–2570.

Wang, L., Mankin, K.R. and Marchin, G.L. (2004) Survival of fecal bacteria in dairy cow manure. *Trans ASAE* **47**, 1239.